

Quantitative cancer stem cell phosphoprotein profiling

Using tandem mass tags and LC-MS/MS

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Introduction

Cancer stem cells (CSCs) are hypothesized to provide a repository of cells that are refractory to chemotherapeutic agents developed for treating differentiated tumor cells.¹ A glioblastoma cancer stem cell line (NSC11) differentiates from a pluripotent state to a progenitor cell-like state when stimulated with WP1193, an inhibitor of STAT3 phosphorylation.² In this study, a new quantitative phosphoproteomics workflow was applied to determine pathways involved in NSC11 differentiation. Knowledge of the signaling process is expected to provide new targets for therapeutic intervention.

Recently, a novel autocrine loop (IL-6/STAT3/HIF1 α) was discovered in pancreatic cancer cells³ (Figure 1A). The objective of this study is to probe how perturbations of this loop induce changes in other pathways in CSCs. IL-6 induces the JAK pathway via the IL-6 receptor and STAT3 is a downstream target.⁴ Activated STAT3 increases HIF1 α (induced by hypoxia) by blocking degradation or enhancing gene transcription.⁵ A comparison of multiple data sets revealed insights as to each factor's contribution to CSC differentiation and how they relate to regulation of other signaling pathways.

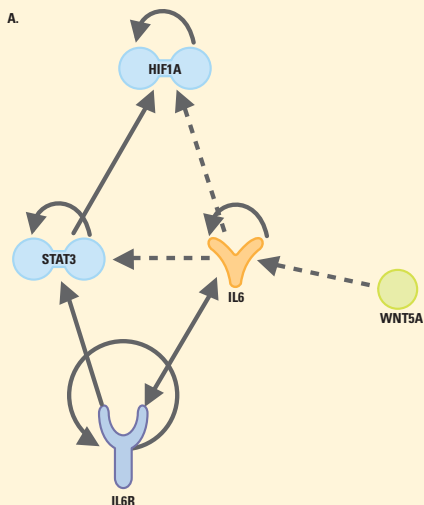


Figure 1. Experimental design. Panel A: Schematic of the IL-6/STAT3/HIF1 α autocrine loop showing direct (—) and indirect (---) interactions between the indicated pathways. (Adapted from Path Designer Software, Ingenuity Systems, Inc.) **Panel B:** NSC11 cancer stem cell treatment strategies that probe the involvement of key players in the IL-6/STAT3/HIF1 α pathway loop. Where indicated, NSC11 cells were treated with the STAT3 phosphorylation inhibitor WP1193 (5 μ M) for 24 hours or IL-6 (10 ng/ml) for 20 minutes before harvest.

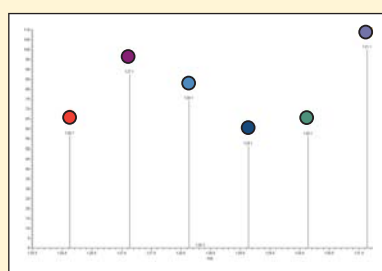
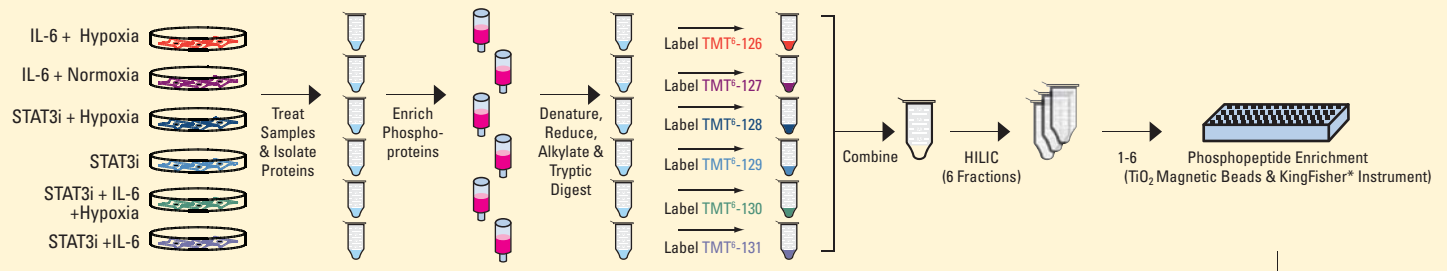
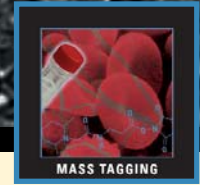
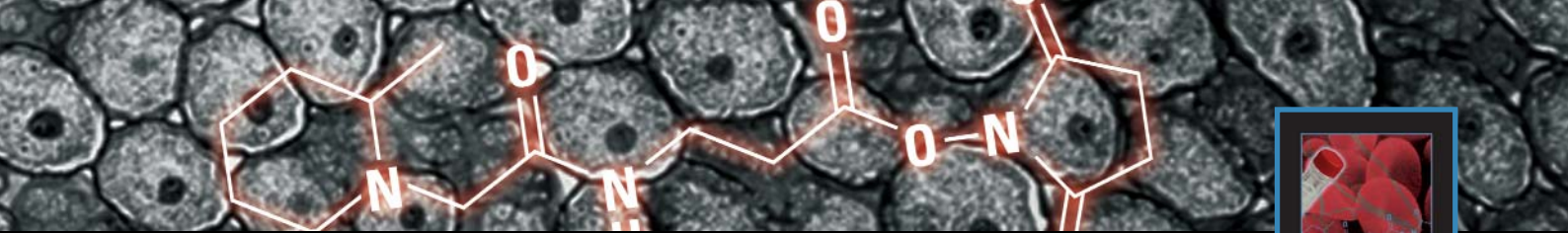
Experimental Design

The treatments were designed to determine the response of NSC11 cells to IL-6, STAT3 inhibition and hypoxia in various combinations (Figure 1B).

NSC11 cells were cultured in serum-free DMEM/F12 medium supplemented with B27, EGF and BFGF. Six different 24-hour treatments of $\sim 2 \times 10^7$ cells (each) were studied, including vehicle control, treatment with IL-6 (10 ng/ml) or WP1193 (5 μ M) and WP1193 plus IL-6 stimulation, and cell culture conditions with normoxia or hypoxia. Cells were harvested and lysed in modified RIPA buffer (50 mM Tris•HCl, pH 7.4; 1% NP-40, 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA) in the presence of Thermo Scientific Halt Protease Inhibitors and Halt Phosphatase Inhibitors. After cell lysis, phosphoproteins were enriched using the Thermo Scientific Pierce Phosphoprotein Enrichment Kit (Product # 90003), and reduced, alkylated and enzymatically digested (Figure 2). Peptides were labeled with Thermo Scientific Isobaric Tandem Mass Tags (TMT[®]) at amino termini and lysine residues. These six labeled peptide samples were combined into one sample, and phosphopeptides were enriched by hydrophilic interaction (HILIC) chromatography and TiO₂-enrichment before analysis with the Thermo Scientific LTQ-Orbitrap Mass Spectrometer. Protein identification was performed with Thermo Scientific Proteome Discoverer Software and the IPI protein database, and relative peptide quantification was performed simultaneously by comparing the abundance ratios of the reporters from the TMT* Tags. Analysis of protein networks was performed using the Ingenuity Pathways Analysis (Ingenuity* Systems, www.ingenuity.com).

B.

NSC11 CSC's	Treatment Conditions for TMT Profiling					
	1	2	3	4	5	6
Growth Condition	Hypoxia	Normoxia	Hypoxia	Normoxia	Hypoxia	Normoxia
Stimulus	IL-6	IL-6			IL-6	IL-6
Inhibitor			STAT3i	STAT3i	STAT3i	STAT3i



MS/MS Quantitation

Gene ID	127	129	130	131	Description	
	126	128	129	130		
NP_024710	1.16	0.76	0.21	0.12	1.0	transcriptional activator protein-1 class 1
NP_054752	1.75	1.24	0.82	0.50	0.28	heat shock protein 90-alpha family class B member 1
NP_054845	1.27	0.89	0.59	0.39	0.25	hydroxyethyl (17-beta)-estradiol synthase 1
NP_056889	1.85	1.27	0.73	0.45	0.26	protein phosphatase, beta subunit isoform 1
NP_056920	1.74	1.19	0.71	0.41	0.24	protein phosphatase, beta subunit isoform 2
NP_056924	1.91	1.42	0.95	0.58	0.33	protein phosphatase, beta subunit isoform 3
NP_056928	0.28	0.21	0.13	0.07	0.04	protein phosphatase, beta subunit isoform 4
NP_057002	1.08	0.72	0.45	0.26	0.15	45S ribosomal protein L32
NP_057009	0.86	0.58	0.35	0.21	0.12	45S ribosomal protein L31
NP_057010	0.83	0.55	0.33	0.2	0.11	45S ribosomal protein L30
NP_057011	0.87	0.57	0.34	0.2	0.11	45S ribosomal protein L29
NP_057012	0.87	0.57	0.34	0.2	0.11	45S ribosomal protein L28
NP_057013	0.87	0.57	0.34	0.2	0.11	45S ribosomal protein L27
NP_057014	0.87	0.57	0.34	0.2	0.11	45S ribosomal protein L26
NP_057015	0.87	0.57	0.34	0.2	0.11	45S ribosomal protein L25
NP_057016	0.87	0.57	0.34	0.2	0.11	45S ribosomal protein L24
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NP_057037	0.87	0.57	0.34	0.2	0.11	45S ribosomal protein L3
NP_057038	0.87	0.57	0.34	0.2	0.11	45S ribosomal protein L2
NP_057039	0.87	0.57	0.34	0.2	0.11	45S ribosomal protein L1
NP_057040	0.87	0.57	0.34	0.2	0.11	45S ribosomal protein L1

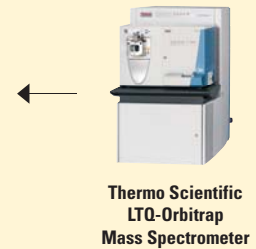
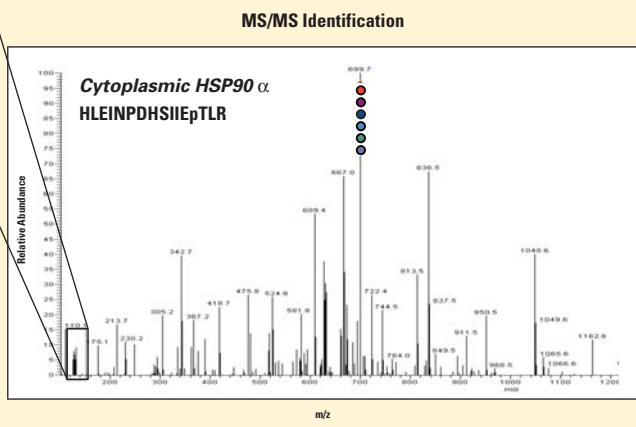


Figure 2. Quantitative phosphoproteomic workflow. The procedure combines phosphoprotein enrichment, isobaric mass tag (TMT) labeling, and phosphopeptide enrichment before mass spectrometry.

* Trademark, see Trademark Index on page 19.

Results and Discussion

More than 200 proteins were quantified in this initial study. The largest number of significant protein changes was observed during hypoxic conditions compared to normoxic conditions with IL-6 stimulation. Functions associated with the quantified phosphoproteins included transcription factors (11%), indicating a significant enrichment of low-abundance proteins (Figure 3).

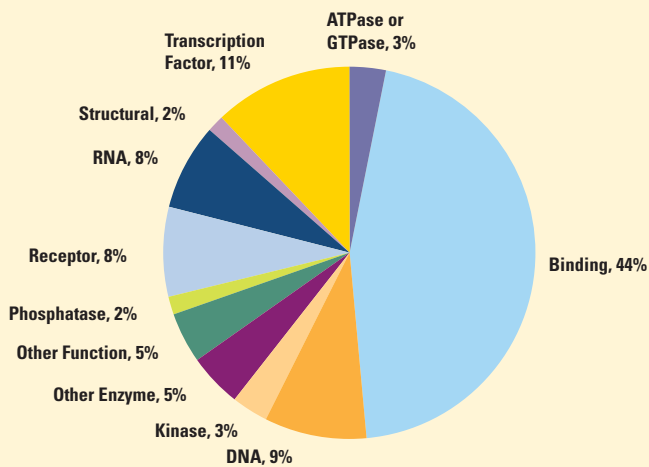


Figure 3. The major protein functions identified in the phosphoprotein- and phosphopeptide-enriched mass spectrometry data set.

In addition to studying changes in protein expression levels, differential phosphorylation at specific sites of the same protein occurs as a result of the various treatments. The high sensitivity of the assay enables detection and quantification of phosphorylated peptides from key, low-abundance proteins such as transcription factors (Figures 4A and B). Because large numbers of changes in peptide phosphorylation patterns were measured in the assay, it will be possible to assign by computational methods kinases that are activated in the intracellular pathways, even if the kinases are not directly measured.

Approximately 15% of the quantified phosphoproteins were linked to the IL-6 pathway (Figure 5). Approximately 70% of the remaining proteins could be assigned to other interlinked networks that were regulated by the experimental treatments, and differential effects of the treatments were observed in those networks and canonical signaling pathways. Of the remaining unassigned data, novel assignments reside, but those need to be further examined with the help of computational experts.

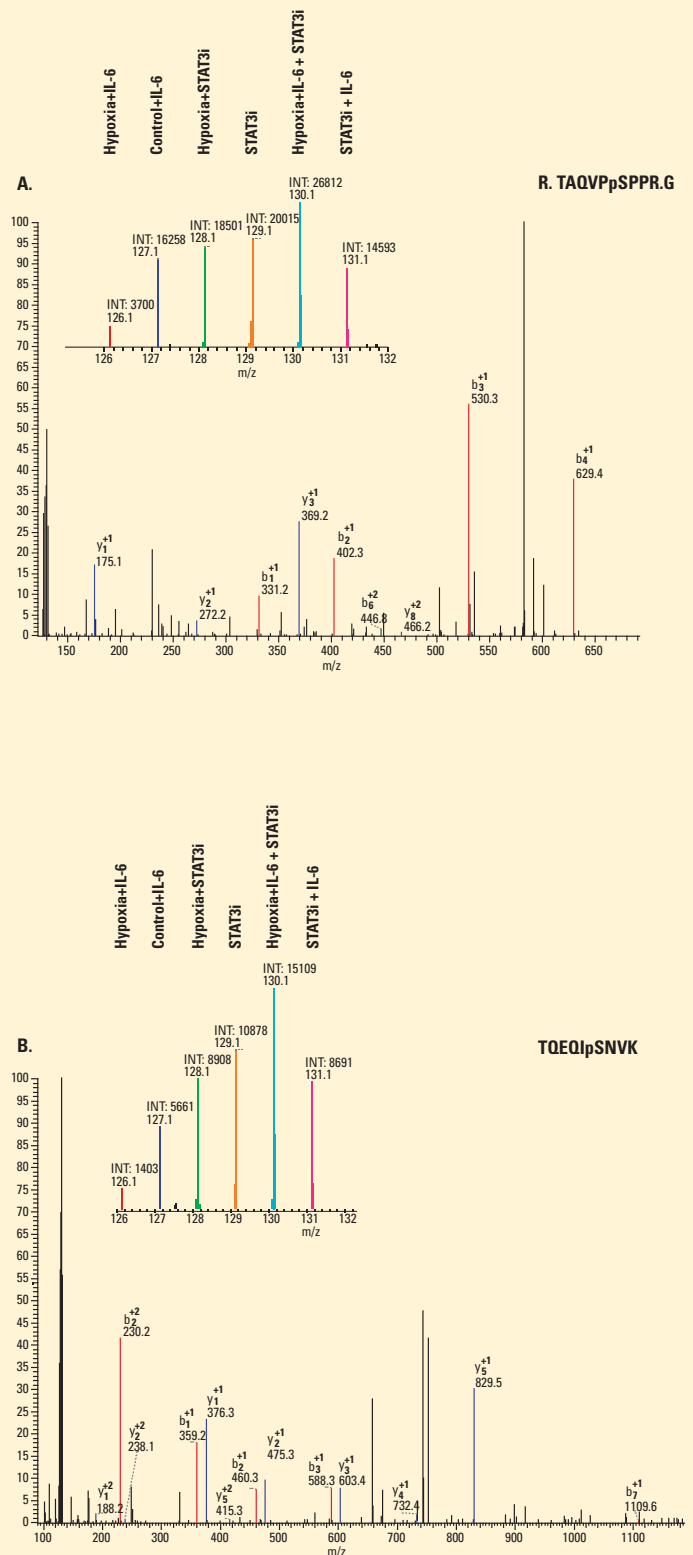


Figure 4. Mass spectrometry peptide and tandem mass tags profiles. **Panel A:** Phospho-apoptotic chromatin condensation inducer 1 protein. **Panel B:** Bcl2-associated transcription factor. Large profiles show the peptide signature. The insets show the mass tag profiles indicating the relative abundance of the respective peptide in each of the six treated samples.

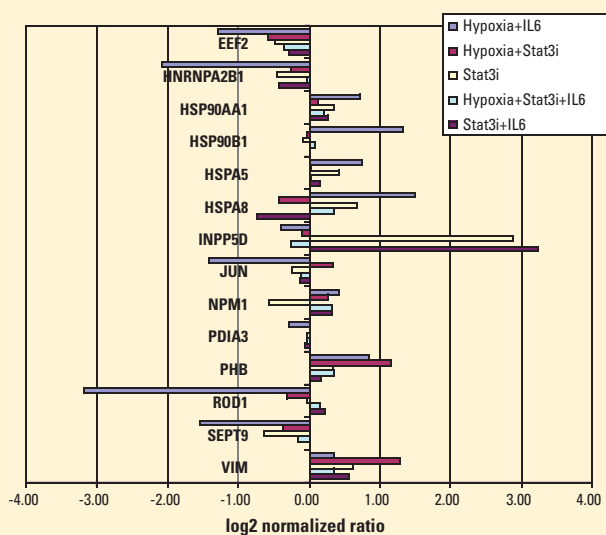
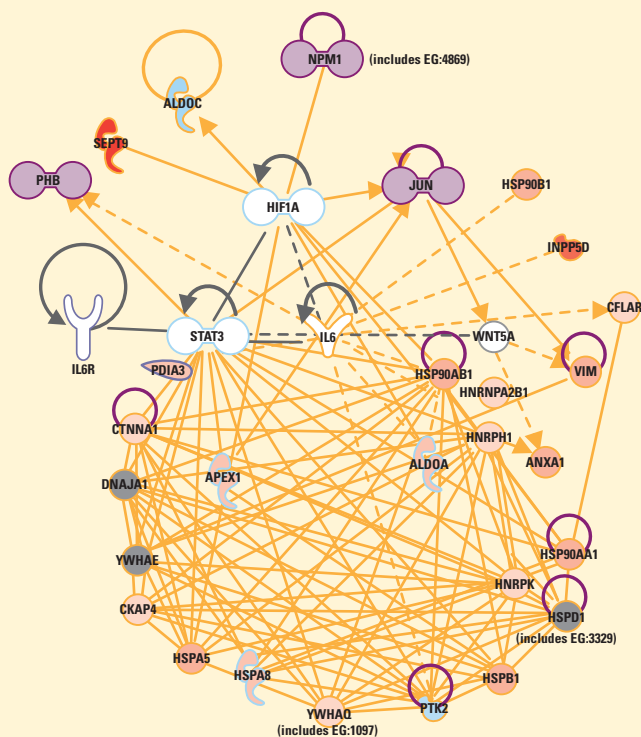
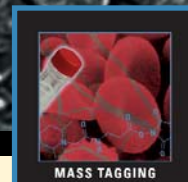
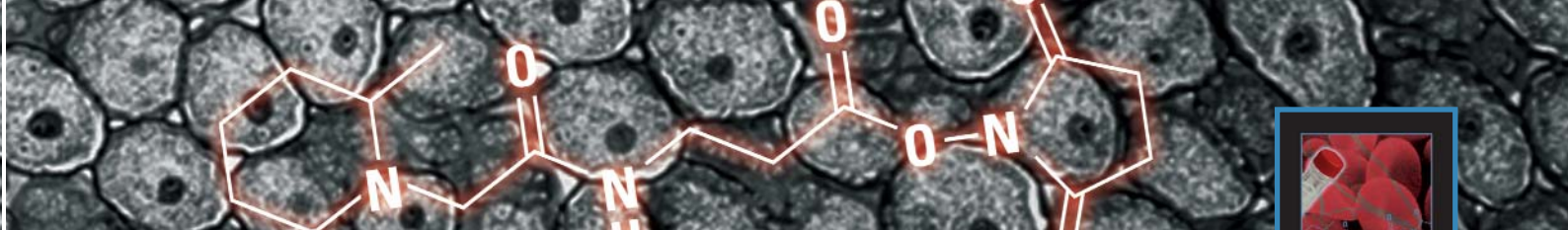


Figure 5. Quantified IL-6/STAT3/HIF1 α signaling partners. **Top panel:** The complex network of identified interacting proteins was superimposed on the original IL-6/STAT3/HIF1 α pathway loop (white symbols). The pink and red coloration indicates proteins identified by mass spectrometry; depth of shading is related to magnitude of the quantified change in expression. Purple symbols represent proteins that were not directly identified but were inferred based on the other proteins/peptides identified. **Bottom panel:** Relative differences in the identified phosphoproteins are expressed as ratios normalized to the normoxia/IL-6 control condition. Positive normalized ratios indicate up-regulation, and negative ratios indicate those proteins down-regulated relative to the normoxia/IL-6 condition.

Summary

Quantification of pathway changes in NSC11 cancer stem cells in response to the six different experimental conditions showed expected pathway changes in the IL6/STAT3/HIF1 α loop but also provided peptide phosphorylation patterns and novel knowledge of other pathways changed in response to the treatments. The assignment of “unmatched” protein responses currently not defined in the literature by high-level statistical analysis will provide further insights into the behavior of the NSC11 cells during differentiation to progenitor-like cells.

References

1. Eyler, C.E. and Rich, J.N. (2008). Survival of the fittest: Cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Oncol* **26**(17):2839-45.
2. Colman, H., et al. (2003). Effect of a small molecule inhibitor of the JAK2/STAT3 pathway on self-renewal of glioblastoma stem cells. *J Clin Oncol* **26** (May 20 supplement).
3. Lang, S.A., et al. (2007). Targeting heat shock protein 90 in pancreatic cancer impairs insulin-like growth factor-1 receptor signaling, disrupts an interleukin-6/signal-transducer and activator of transcription 3/hypoxia-inducible factor-1 α autocrine loop, and reduces orthotopic tumor growth. *Clin Cancer Res* **13**(21):6459-68.
4. Lee, T.L., et al. (2006). Epigenetic modification of SOCS-1 differentially regulates STAT3 activation in response to interleukin-6 receptor and epidermal growth factor receptor signaling through JAK and/or MEK in head and neck squamous cell carcinomas. *Mol Cancer Ther* **5**(1):8-19.
5. Jung, J.E., et al. (2005). STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells. *FASEB J* **19**(10):1296-8.

Ordering Information

Product #	Description	Pkg. Size
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90064	TMTsixplex* Isobaric Mass Tagging Kit Labeling Reagents for Multiplexed and Absolute Protein Quantification	Kit
90065	TMTduplex Label Reagent Set Labeling Reagents for Multiplexed and Absolute Protein Quantification	Kit
90066	TMTsixplex Label Reagent Set Labeling Reagents for Multiplexed and Absolute Protein Quantification	Kit
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88812	Pierce Magnetic Titanium Dioxide Phosphopeptide Enrichment Kit, Trial Size	Kit
90003	Pierce Phosphoprotein Enrichment Kit	Kit

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